

Colonization of broilers by *Campylobacter jejuni* internalized within *Acanthamoeba castellanii*

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Abstract Although *Campylobacter* survives within amoeba in-vitro, it is unknown if intra-amoeba *Campylobacter jejuni* can colonize broilers. Five groups of 28 day-of-hatch chicks were placed into separate isolators. Groups (1) and (2) were challenged with page's amoeba saline (PAS), and disinfected planktonic *C. jejuni* NCTC 11168, respectively. Groups (3), (4) and (5) were challenged with a *C. jejuni* positive control, *C. jejuni* in PAS, and intra-amoeba *C. jejuni*, respectively. After 1, 3, 7 and 14 days post challenge, seven birds from each unit were examined for *C. jejuni* colonization. For the first time we report that intra-amoeba *C. jejuni* colonized broilers.

Keywords *Campylobacter jejuni* · Broilers · Drinking water · Protozoa · Epidemiology

Introduction

Campylobacter jejuni is a gram-negative zoonotic bacterium which is the most common cause of bacterial food poisoning in the developed world (Callicott et al. 2006). In developed countries, by the time poultry are one-month-old, roughly half of flocks will contain *C. jejuni* (Snelling et al. 2005a). Controlling *Campylobacter* carriage in the poultry reservoir might have a measurably beneficial effect on human disease incidence (ACMSF 2004).

Most evidence suggests that vertical transmission is not a significant source of contamination (Callicott et al. 2006), and poultry are colonized from environmental sources, e.g., drinking water, where protozoa are also present (Snelling et al. 2005b, 2006a). Previously, using a combination of selective enrichments, biochemical assays and molecular identification tests, we detected *C. jejuni* and a variety of protozoa in the drinking water of intensively reared broilers (Snelling et al. 2005b, 2006a). Broilers were only colonized with *C. jejuni* if it was also present in their drinking water, illustrating the importance of water as a *C. jejuni*'s source, where interactions would likely occur with protozoa (Snelling et al. 2005b, 2006a). During in-vitro assays, which involved co-culturing *Campylobacter* and *Acanthamoeba castellanii*, we found that *C. jejuni* remained culturable for significantly longer (upto 36 h) in the presence of *A. castellanii*, than when they were in purely, a planktonic state (Snelling et al. 2005b). Internalized *Campylobacter* were also significantly more resistant to an iodine-based industrial disinfectant. Collectively these results suggested that protozoa in broiler drinking water increased the potential of *C. jejuni* to colonize broilers (Snelling et al. 2005b, 2006a, b). In the present study we investigated the ability of intra-amoeba *C. jejuni* (within *A. castellanii*) to colonize broilers.

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Materials and methods

Co-culture preparation

For all experiments, *C. jejuni* NCTC 11168 (the type strain) and *A. castellanii* CCAP 1501/10 (Culture Collection of Algae and Protozoa, Oban, UK; identical to ATCC 30868) were used. For the present study, *A. castellanii* was selected because it can be grown axenically, significantly prolong *C. jejuni* viability in low nutrient conditions, and provide bacteria with protection from halogen disinfectants (Drozanski 1978; King et al. 1988; Snelling et al. 2005b). *C. jejuni* were grown on Preston agar (Oxoid, Basingstoke, Hampshire, UK) under microaerobic conditions for 24 h at 42 °C using CampyGen (Oxoid) gas packs in 3.5-l anaerobic jars (Oxoid). *C. jejuni* were then resuspended in page's amoeba saline solution (PAS; CCAP), enumerated using culturable plate counts and optical densities (OD) were measured at 600 nm ($OD\ 0.4:1.3 \times 10^6\ cfu\ ml^{-1}$). *A. castellanii* were grown for three days in proteose peptone glucose broth (PPG, CCAP) with penicillin-streptomycin [(50 U ml^{-1} penicillin G and 50 $\mu g\ ml^{-1}$ streptomycin (Invitrogen, Paisley, UK)] at 25°C to achieve population densities of around $10^6\ cells\ ml^{-1}$ (Snelling et al. 2005b). Co-cultures were produced using methods previously established by Snelling et al. (2005b). Briefly, *A. castellanii* was gravity filtered by using membrane filters (0.8 μm pore size; Whatman, Maidstone, England) to remove broth. Cells were then resuspended in 1:1 dilutions of PPG and PAS (CCAP; total volume, 10 ml) and incubated at 25°C overnight (Hatzis et al. 1993; Snelling et al. 2005b). Cells were filtered again (as described above), rinsed with 10 ml PAS to remove traces of antibiotics, resuspended in 10 ml of PAS solution and enumerated using direct hemocytometer counts. Co-cultures of *A. castellanii* and *C. jejuni* cells were obtained by adding 0.25 ml of *C. jejuni* suspension to *A. castellanii* in PAS solution, to obtain a 1:10 ratio of protozoa:*C. jejuni* (10 ml final volume). Co-cultures were then incubated for 3 h at 25°C, the same ambient temperature at which intensively reared broilers are reared.

Chlorination assays

To obtain internalized *C. jejuni*, a chlorination assay based on previous studies by King et al. (1988) and Snelling et al. (2005b) was used. All of the following assays were performed in triplicate. To standardize experimental protocols it was necessary to perform a variety of previously described preliminary experiments (Snelling et al. 2005b). Firstly, it was confirmed that after exposure to 1 ml of 10% sodium thiosulfate (STS; Sigma, Dorset, UK) no significant effects ($P > 0.05$) on the growth of *C. jejuni* and *A. castellanii* were observed (data not shown). No significant effects

($P > 0.05$) on *C. jejuni* viability was observed. After 15 s of sonication (50 W) using a probe tip sonicator, which completely disrupted *A. castellanii* trophozoites (data not shown). Exposure to 0.1 ml of a 1:100 dilution of 10% sodium hypochlorate (Sigma) for a contact time of 1 min, followed by neutralization with 1 ml of 10% STS, killed planktonic *C. jejuni* (0.25 ml of *C. jejuni* suspension and 9.75 ml of PAS), but had no significant effect ($P > 0.05$) on the growth of *A. castellanii* in PAS at 25°C (data not shown) (King et al. 1988; Snelling et al. 2005b). Consequently this treatment was used for all subsequent co-culture disinfection assays to obtain internalized *C. jejuni*.

To quantify the number of internalized *C. jejuni* which survived disinfection, co-cultures were prepared as described above, disinfected, neutralized, rinsed with excess PAS, sonicated, and culturable *C. jejuni* counts (48 h at 42°C on Preston agar) were then performed (Snelling et al. 2005b). Individual colonies were streaked for purity onto Preston agar plates and grown microaerobically for 24 h at 42°C. Gram staining and biochemical testing using Mast ID Camp Identification Systems (Mast Diagnostics, Bootle, UK) were performed to check for vibroid morphology and for hippurate hydrolysis and indoxyl acetate and urease activity, respectively. To determine whether culturable counts were significantly different ($P < 0.05$) between *C. jejuni* in a planktonic state compared to when they were inside *A. castellanii*, data were compared using SPSS 11.0 software (SPSS, Inc., Chicago, IL, USA) and the Bonferroni (one-way analysis of variance) multiple comparison test.

Broiler challenge model

The broiler challenge trial was conducted at PMSRU, ARS, USDA, under the guidelines of the Institutional Animal Care and Use Committee. All steps for growing *A. castellanii* and obtaining internalized *C. jejuni* were the same as described above, apart from the replacement of Preston media with Campy-Cefex media (Stern et al. 1992), and were also carried out at the PMSRU. Five groups of 28 *C. jejuni*-free day-of-hatch birds were placed into separate isolation units with sterile feed, water and litter. Chicks in each unit were then orally challenged with 0.2 ml of (A) PAS, (B) *C. jejuni* incubated for 3 h at 25°C in PAS, which had been disinfected and neutralized (no culturable CFUs ml^{-1}), (C) standard *C. jejuni* positive control (7.6×10^6 culturable CFUs ml^{-1}), (D) *C. jejuni* in PAS incubated for 3 h at 25°C (8.4×10^4 culturable CFUs ml^{-1}), and (E) internalized *C. jejuni* in *A. castellanii* (1.7×10^4 culturable CFUs ml^{-1}). As a precaution, in case intra-amoeba *C. jejuni* and *C. jejuni* which had been incubated in PAS for 3 h at 25°C did not colonize broilers, oral challenge (C) with the highest levels of *C. jejuni* was used

as an additional control to verify the colonization potential of the selected strain. After 1, 4, 7 and 14 days post challenge seven birds from each unit were euthanized by cervical disarticulation (Stern et al. 2006). Ceca of the individual animals were aseptically dissected. Enumeration of *C. jejuni* was accomplished by diluting 1 g of cecal content per 9 ml of phosphate-buffered saline (pH 7.2) solution (Stern 1994; Stern et al. 2006). Tenfold serial dilutions were made and 0.1-ml portions were surface plated onto Campy-Cefex agar plates (Stern et al. 1992). Plates were incubated at 42°C for 48 h under a microaerobic atmosphere (Stern et al. 2006), and characteristic *Campylobacter* colonies were counted after confirmation using phase contrast microscopy.

Results and discussion

Internalized *C. jejuni* are protected from chlorination and colonize broilers

The aim of our experiment was to ascertain if intra-amoeba *C. jejuni* could colonize broilers. Essential preliminary studies showed that significantly more culturable intra-amoeba *C. jejuni* ($P < 0.05$) were recovered after exposure to a chlorination regime which killed equal numbers of culturable, planktonic *C. jejuni* (Fig. 1). Importantly, after 1, 3, 7 and 14 days post challenge none of the broilers challenged with PAS and disinfected planktonic *C. jejuni* (negative controls) were colonized with *C. jejuni*. Broilers were colonized after challenge with both of the positive controls; standard *C. jejuni* control and *C. jejuni* incubated in PAS for 3 h at 25°C. Importantly, broilers were also colonized for 1, 4, 7 and 14 days post challenge by intra-amoeba *C. jejuni* (Table 1). Rinsing with excess PAS buffer removes all surviving external *C. jejuni* (Fig. 1). This was confirmed by both viable counts and fluorescence microscopy; fluorescein-iso-thiocyanate (FITC)-labeled internal *C. jejuni*, and DAPI 4',6'-diamidino-2-phenylindole labeled external *C. jejuni* (Snelling et al. 2005b). It is important to note that the four challenge controls were present, to be absolutely certain that any cecal colonization resulting from challenge (E) definitely only resulted from intra-amoeba *C. jejuni*. During oral challenge sample preparation, although equal amounts of *C. jejuni* were added to samples (B), (D), (E) due to the necessary experimental steps, large variations occurred in the resulting concentrations of culturable cells in challenge samples, e.g. culturable cells from samples C and D were ~ 450 and fivefold higher, respectively, than from sample E (intra-amoeba *C. jejuni*: 1.7×10^4 CFUs ml^{-1}). Therefore, relative comparisons of colonization data cannot be made.

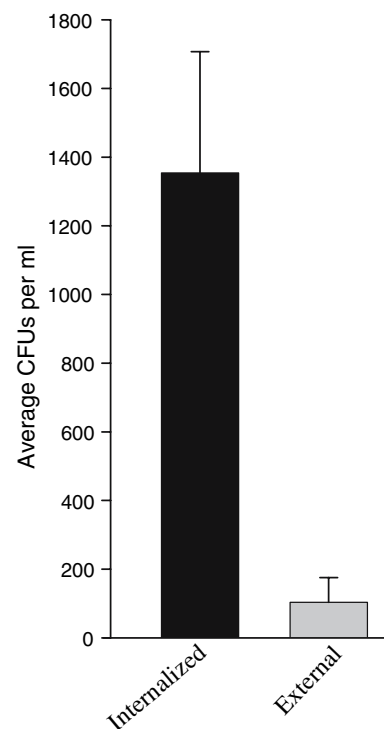


Fig. 1 Significantly more intra-amoeba *C. jejuni* survive chlorination. *A. castellanii* (CCAP 1501/10) were each grown for 3 days before being co-cultured for 3 h at 25°C (1:10 ratio) with *C. jejuni* NCTC 11168 in PAS solution. A 1:100 dilution of 10–13% sodium hypochlorite (1-min contact time) was then used to kill planktonic *C. jejuni*, followed by neutralization using STS, gravity filtration (0.8 μm pore size), rinsing, resuspension in PAS, sonication (15 s at 50 W), and culturable *Campylobacter* counts were performed. Results are presented as the average (performed in triplicate) number of culturable *C. jejuni* recovered ml^{-1} of PAS and error bars are standard deviations

Table 1 The average cecal colonization of broilers following challenge with *C. jejuni* NCTC 11168

Number of days post-challenge	Log of the average number of <i>C. jejuni</i> NCTC 11168 present in broiler ceca g^{-1}		
	Standard <i>C. jejuni</i>	<i>C. jejuni</i> in PAS	Intra-amoeba <i>C. jejuni</i>
1	3.84 3 ^a , 1 ^b	4.04 5 ^a , 0 ^b	4.63 1 ^a , 1 ^b
4	6.72 5 ^a , 0 ^b	5.87 3 ^a , 1 ^b	6.19 2 ^a , 0 ^b
7	7.18 2 ^a , 0 ^b	7.36 3 ^a , 0 ^b	7.16 1 ^a , 0 ^b
14	> 7.16 1 ^a , 5 ^b	> 8.00 1 ^a , 6 ^b	TNTC 0 ^a , 7 ^b

^a Number of birds out of 7 not colonized with *C. jejuni*

^b Number of birds out of 7 with *C. jejuni* colonization levels too numerous to count (TNTC: $> 10^8$ CFUs g^{-1})

Campylobacter epidemiological implications

Previously, we hypothesized that *C. jejuni* persistence and disinfection resistance in drinking water systems could be increased following interactions with protozoa, indicating that protozoa may serve as a non-vertebrate reservoir for *C. jejuni* in the environment (Snelling et al. 2006b). In this study we are the first to demonstrate that internalized *C. jejuni* within *A. castellanii* colonized broilers. Therefore protozoa could act as ‘Trojan horses’ in broiler drinking water, bringing hidden *C. jejuni* within poultry, resulting in their colonization (Greub and Raoult 2004). The interaction between protozoa and bacteria has been examined mostly from the perspective of predator-prey relationship (Brandl et al. 2005). The role of protozoa in the virulence of food-borne pathogens and their persistence in the environment is an important and emerging area of research (Brandl et al. 2005; Steinert et al. 1994). It has been shown by several studies that bacteria are capable of surviving within the protozoa and this protection, due to the internalization of bacteria, has been documented (Snelling et al. 2006b).

Conclusions

For the first time we report that intra-amoeba *C. jejuni* colonized broilers. The biology of interactions between *A. castellanii* spp. and *C. jejuni* urgently needs to be examined further. It would also be essential to examine potential *Campylobacter* interactions with the protozoan groups, including flagellates and alveolates, we previously detected in the drinking water of intensively reared poultry (Snelling et al. 2006a). In poultry rearing facilities the adoption of rigorous cleaning regimes with disinfectants has been used to try to reduce waterborne infection with limited success and bacteria which are sensitive to disinfectants in vitro still can persist in water distribution systems (Fields and Swerdlow 1999). *Campylobacter* interactions with protozoa may partially explain observations that the chlorination of broiler drinking water had no effect on *C. jejuni* colonization (Newell and Fearnley 2003; Stern et al. 2002). *C. jejuni* and protozoa in biofilms found in animal production watering systems may also play a role in the colonization of broilers (Reaser et al. 2007). Increased information of the microbial diversity in the water supplies of broilers could be invaluable for further epidemiology data to help in controlling both animal and human pathogens in the poultry industry. The biology of interactions between different *A. castellanii* spp., e.g. *A. polyphaga* (Axelsson-Olsson et al. 2005), and other species of protozoa, and *C. jejuni* urgently needs to be examined further to increase current understanding and

accurately assess the true potential of protozoa being an environmental *Campylobacter* reservoir.

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References

- Advisory Committee on the Microbiological Safety of Food (ACMSF) (2004) Second report on *Campylobacter*. <http://www.food.gov.uk/multimedia/pdfs/acmsfcampyloreport.pdf>
- Axelsson-Olsson D, Waldenstrom J, Broman T, Olsen B, Holmberg M (2005) Protozoan *Acanthamoeba polyphaga* as a potential reservoir for *Campylobacter jejuni*. Appl Environ Microbiol 71:987–992
- Brandl MT, Rosenthal BM, Haxo AF, Berk SG (2005) Enhanced survival of *Salmonella enterica* in vesicles released by a soil-borne *Tetrahymena* species. Appl Environ Microbiol 71:1562–1569
- Callicott KA, Friethriksdottir V, Reiersen J, Lowman R, Bisailon JR, Gunnarsson E, Berndtson E, Hiatt KL, Needleman DS, Stern NJ (2006) Lack of evidence for vertical transmission of *Campylobacter* spp. in chickens. Appl Environ Microbiol 72:5794–5798
- Drozanski W (1978) Activity and distribution of bacteriolytic *N*-acetyl-muramidase during growth of *Acanthamoeba castellanii* in axenic culture. Acta Microbiol Pol 27:243–256
- Fields PI, Swerdlow DL (1999) *Campylobacter jejuni*. Clin Lab Med 19:489–504
- Greub G, Raoult D (2004) Microorganisms resistant to free-living amoebae. Clin Microbiol Rev 17:413–433
- Hatzis C, Sreenc F, Fredrickson AG (1993) Feeding heterogeneity in ciliate populations effects of culture age and nutritional status. Biotechnol Bioeng 43:371–380
- King CH, Shotts EB Jr, Wooley RE, Porter KG (1988) Survival of coliforms and bacterial pathogens within protozoa during chlorination. Appl Environ Microbiol 54:3023–3033
- Newell DG, Fearnley C (2003) Sources of *Campylobacter* colonization in broiler chickens. Appl Environ Microbiol 69:4343–4351
- Reaser RJ, Medler RT, Billington SJ, Jost BH, Joens LA (2007) Characterization of *Campylobacter jejuni* biofilms under defined growth conditions. Appl Environ Microbiol 73:1908–1913
- Snelling WJ, Moore JE, Dooley JSG (2005a) The colonization of broilers with *Campylobacter*. Worlds Poult Sci J 61:655–662
- Snelling WJ, McKenna JP, Lecky DM, Dooley JS (2005b) Survival of *Campylobacter jejuni* in waterborne protozoa. Appl Environ Microbiol 71:5560–5571
- Snelling WJ, McKenna JP, Hack CJ, Moore JE, Dooley JSG (2006a) An examination of the diversity of a novel *Campylobacter* reservoir. Arch Microbiol 186:31–40
- Snelling WJ, McKenna JP, Moore JE, Dooley JSG (2006b) Bacterial-protozoa interactions; an update on the role these phenomena play towards human illness. Microbes Infect 8:578–587
- Steinert M, Ott MM, Luck PC, Tannich E, Hacker J (1994) Studies on the uptake and intracellular replication of *Legionella pneumophila* in protozoa and in macrophage-like cells. FEMS Microbiol Ecol 15:299–307

- Stern NJ, Wojton B, Kwiatek K (1992) A differential-selective medium and dry ice-generated atmosphere for recovery of *Campylobacter jejuni*. J Food Prot 55:514–517
- Stern NJ (1994) Mucosal competitive exclusion to diminish colonization of chickens by *Campylobacter jejuni*. Poult Sci 73:402–407
- Stern NJ, Robach MC, Cox NA, Musgrove MT (2002) Effect of drinking water chlorination on *Campylobacter* spp. colonization of broilers. Avian Dis 46:401–404
- Stern NJ, Svetoch EA, Eruslanov BV, Perelygin VV, Mitsevich EV, Mitsevich IP, Pokhilenko VD, Levchuk VP, Svetoch OE, Seal BS (2006) Isolation of a *Lactobacillus salivarius* strain and purification of its bacteriocin, which is inhibitory to *Campylobacter jejuni* in the chicken gastrointestinal system. Antimicrob Agents Chemother 50:3111–3116